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Mono- and Disubstituted-3,8-diazabicyclo[3.2.1]octane Derivatives as Analgesics Structurally Related to Epibatidine: Synthesis, Activity, and Modeling

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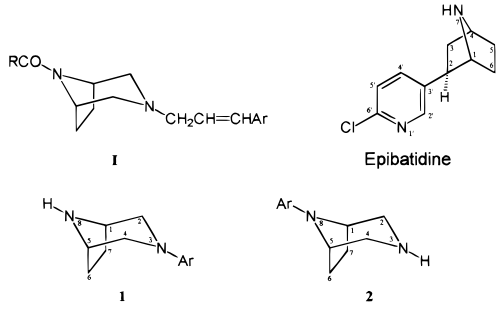
A series of 3,8-diazabicyclo[3.2.1]octanes substituted either at the 3 position (compounds **1**) or at the 8 position (compounds **2**) by a chlorinated heteroaryl ring were synthesized, as potential analogues of the potent natural analgesic epibatidine. When tested in the hot plate assay, the majority of the compounds showed significant effects, the most interesting being the 3-(6-chloro-3-pyridazinyl)-3,8-diazabicyclo[3.2.1]octane (**1a**). At a subcutaneous dose of 1 mg/kg, **1a** induced a significant increase in the pain threshold, its action lasting for about 45 min. **1a** also demonstrated good protection at a dose of 5 mg/kg in the mouse abdominal constriction test, while at 20 mg/kg it completely prevented the constrictions in the animals. Administration of naloxone (1 mg/kg ip) did not antagonize its antinociception while mecamylamine (2 mg/kg ip) did, thus suggesting the involvement of the nicotinic system in its action. Binding studies confirmed high affinity for the $\alpha_4\beta_2$ nAChR subtype ($K_i = 4.1 \pm 0.21$ nM). nAChR functional activity studies on three different cell lines showed that **1a** was devoid of any activity at the neuromuscular junction. Finally, due to the analogy in their pharmacological profile with that of epibatidine, compounds were compared from a structural and conformational point of view through theoretical calculations and high-field ^{1}H NMR spectroscopy. Results indicate that all of them present one conformation similar to that of epibatidine.

Introduction

Epibatidine (Chart 1) was first isolated by Daly and co-workers[1] from the skin of the ecuadoran poison frog *Epipedobates tricolor* and its structure determined as *exo*-2-(6-chloro-3-pyridyl)-7-azabicyclo[2.2.1]heptane. The alkaloid was shown to be 200-fold more potent than morphine in the hot plate test with a non-opioid and non-cholinergic muscarinic mechanism of action, as evidenced by the inability of naloxone and scopolamine to prevent it.[1−3] On the contrary, its antinociception was fully blocked by the nicotinic antagonists mecamylamine and dihydro-β-erythroidine.[2,3] Epibatidine displaced [^{3}H]cytisine from $\alpha_4\beta_2$ nAChR in rat brain membranes with high potency ($K_i = 0.043$ nM),[2,4] while it exhibited much lower affinity for the muscarinic ligand binding site ([^{3}H]−*N*-methylscopolamine) in rat cortical membranes.[3]

In the course of our studies on novel analgesics, structurally unrelated to morphine, we developed a class of 3,8-disubstituted-3,8-diazabicyclo[3.2.1]octanes (**I**) provided with significant affinity and selectivity for the μ-opioid receptors and potent analgesic activity in the hot plate test.[5] The presence in epibatidine of the 7-azabicyclo[2.2.1]heptane system prompted us to verify if the skeleton of the diazabicyclooctane could be a proper substrate to develop a new series of epibatidine analogues (**1**), provided with similar analgesic properties and mechanism of action.

Chart 1



1, 2	Ar
a	6-chloro-3-pyridazinyl
d	6-chloro 2-methyltio-4-pyrimidinyl
e	2-chloro-4-pyrimidinyl
f	4-chloro-2-pyrimidinyl
g	6-chloro-4-pyrimidinyl
h	5-chloro-3-pyrazinyl
i	3-chloro-2-pyridyl
j	6-chloro-2-pyridyl

Compounds **1** and their isomers **2** were synthesized and tested for their antinociceptive activity and nicotine

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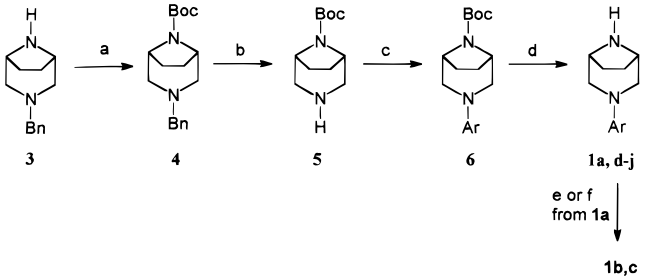
Scheme 1[a]



[a] (a) Di-*tert*-butyl dicarbonate/CH_2Cl_2; (b) 10% Pd−C/H_2; (c) ArCl/toluene/TEA/Δ; (d) Et_2O/HCl; (e) $(CH_3CH_2CO)_2O/CH_2Cl_2$/Δ; (f) C_2H_5I/K_2CO_3/acetone/Δ. Ar for **1** and **2**: **a**, 6-chloro-3-pyridazinyl; **d**, 6-chloro-2-(methylthio)-4-pyrimidinyl; **e**, 2-chloro-4-pyrimidinyl; **f**, 4-chloro-2-pyrimidinyl; **g**, 6-chloro-4-pyrimidinyl; **h**, 5-chloro-3-pyrazinyl; **i**, 3-chloro-2-pyridyl; **j**, 6-chloro-2-pyridyl.

receptor binding affinity. Moreover, they were investigated from a structural and conformational point of view through theoretical calculations and high-field ^1H NMR spectroscopy.

Chemistry

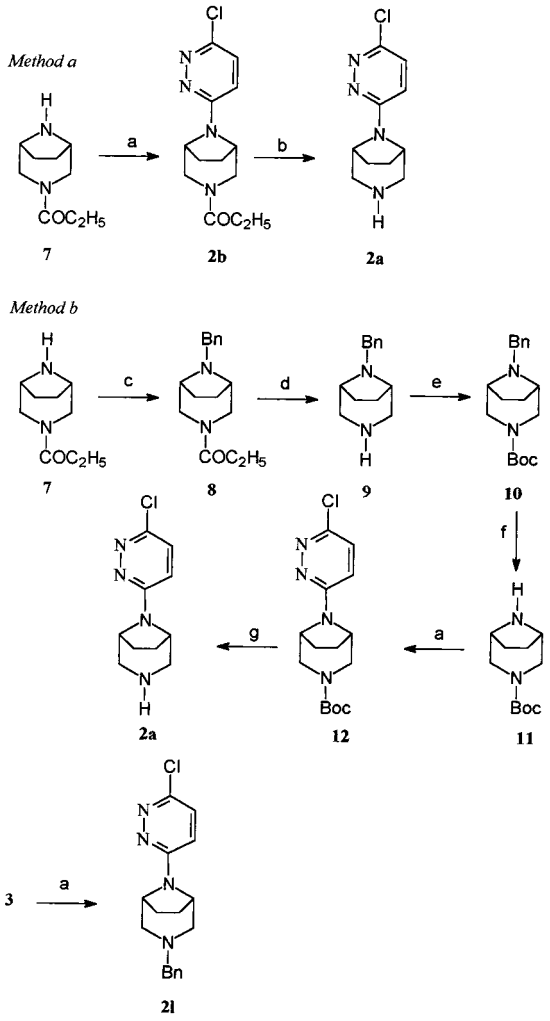
The key intermediate in the synthesis of compounds **1** was the previously reported **3**,[6] which was first protected at N_8 with di-*tert*-butyl dicarbonate in dichloromethane (compound **4**) and then transformed into **5** by catalytic debenzylation. Treatment of the latter with an equimolar amount of the appropriate heterocycle in refluxing toluene and in the presence of equimolar triethylamine gave the Boc derivatives **6**, which were finally deprotected by stirring with a solution of hydrochloric acid in diethyl ether to give the desired **1a,d−j**, as their hydrochlorides. Compounds **1b,c** could easily be prepared from **1a** by condensing with propionic anhydride or iodoethane, respectively (Scheme 1).

To prepare the isomers **2a,b**, the known **7**[7] was condensed with 3,6-dichloropyridazine to form **2b**, followed by alkaline hydrolysis to give **2a** (Scheme 2, method a). Though in the case of **2a** the chlorine in position 6 of the pyridazine ring was resistant to the alkaline medium,[8,9] to avoid a possible replacement of the halogen by the hydroxy group on a different substrate, a more general synthetic pathway was devised, as depicted in method b). Accordingly, **7** was reacted with benzyl chloride to give **8**, which was hydrolyzed in acidic medium to **9**, protected by Boc (**10**), debenzylated (**11**), condensed with 3,6-dichloropyridazine (**12**), and finally converted into **2a** (as its hydrochloride) by stirring overnight with a solution of hydrochloric acid in diethyl ether. It should be noted that, though a much greater number of steps were required in method b with respect to method a, the final yields were almost comparable. Finally, **2l** was easily obtained by condensing **3** with 3,6-dichloropyridazine in refluxing toluene. See Table 1 for data of **1** and **2**.

Pharmacology

All the compounds were tested in vivo in the mouse hot plate test (thermal stimulus) in order to determine their potential analgesic potency and efficacy. They were used at doses unable to modify normal behavior. The integrity of motor coordination was evaluated by the rota-rod test. Data are reported in Tables 2 and 3

Scheme 2[a]



[a] (a) 2,6-Dichloropyridazine/toluene/TEA; (b) 2 N NaOH/Δ; (c) $BnCl/K_2CO_3$/acetone/Δ; (d) 4 N HCl/Δ; (e) di-*tert*-butyl dicarbonate/CH_2Cl_2; (f) 10% Pd−C/H_2; (g) Et_2O/HCl.

and Figures 1−3. Binding toward brain nicotinic receptors as well as nAChR functional activity was also examined.

Molecular Modeling

Epibatidine and compounds **1a,d−j** and **2a**, in their protonated form, were submitted to a modeling study with the aid of theoretical calculations and high-field ^1H NMR spectroscopy. The preferred conformations and the heats of formation of all the compounds were determined using a full geometry optimization carried out at the RHF level with the semiempirical AM1 method[10] implemented in the HyperChem program.[11] All the degrees of conformational freedom were examined, in particular rotation of the aryl moiety and chair−boat interconversion in the case of **1** and **2**. Figure 4 shows the 3D plots and the calculated heats of formation of the significant conformers of epibatidine and compounds **1a** and **2a**, while Table C (see Supporting Information) reports their selected geometrical and conformational features.

Results and Discussion

Analgesic Activity. The data reported in Table 2 show that, at the highest tested concentration (25 mg/

Table 1. Physicochemical Properties of Compounds **1** and **2**

1 **2**

compd	R	Ar	% yield	mp, °C	formula
1a	H	6-chloro-3-pyridazinyl	46	>240[a]	$C_{10}H_{13}ClN_4$
1b	COEt	6-chloro-3-pyridazinyl	85[b]	132	$C_{13}H_{17}ClN_4O$
1c	Et	6-chloro-3-pyridazinyl	60[b]	210−213	$C_{12}H_{17}ClN_4$
1d	H	6-chloro-2-(methylthio)-4-pyrimidinyl	43	>275[a]	$C_{11}H_{15}ClN_4S$
1e	H	2-chloro-4-pyrimidinyl[c]	21	>290[a]	$C_{10}H_{13}ClN_4$
1f	H	4-chloro-2-pyrimidinyl[c]	19	>280[a]	$C_{10}H_{13}ClN_4$
1 g	H	6-chloro-4-pyrimidinyl	43	165−168[a]	$C_{10}H_{13}ClN_4$
1h	H	5-chloro-3-pyrazinyl	39	165−168[a]	$C_{10}H_{13}ClN_4$
1i	H	3-chloro-2-pyridyl	48	220−225[a]	$C_{11}H_{14}ClN_3$
1j	H	6-chloro-2-pyridyl	50	>250[a]	$C_{11}H_{14}ClN_3$
2a	H	6-chloro-3-pyridazinyl	58[d]	dec[a]	$C_{10}H_{13}ClN_4$
2b	COEt	6-chloro-3-pyridazinyl	50	171−172	$C_{13}H_{17}ClN_4O$
2l	Bn	6-chloro-3-pyridazinyl	48	192−194	$C_{17}H_{19}ClN_4$

[a] As the hydrochloride. [b] From **1a**. [c] See ref 24. [d] Method b, Scheme 2.

kg), the majority of the monosubstituted compounds **1** induced a significant increase in the mouse pain threshold (hot plate test), when injected subcutaneously. At lower doses (10−15 mg/kg) significant activity was still seen in several derivatives (**1a,d−g,j**). The rota-rod test (see Table B in Supporting Information) showed that none of them caused any visible change in the normal behavior of the animals. The most interesting compound was the 3-(6-chloro-3-pyridazinyl)-3,8-diazabicyclo-[3.2.1]octane (**1a**), which retained analgesic potency still at 1 mg/kg (see Figure 1). Its analgesic action peaked after 15−30 min and remained almost unchanged after 45 min. In addition, **1a** exhibited the highest ratio (30) between the maximal nontoxic dose (MNTD) and the minimal analgesic dose (MAD). For the other active drugs this value was never higher than 5 (see Table 3). Insertion of an ethyl group at position 8 of **1a** gave a much weaker compound (**1c**), while substitution at the same position by a propionyl group (**1b**) brought about a complete loss of activity. Furthermore, shifting the chloropyridazinyl ring from the 3 to the 8 position (compound **2a**) led to a less-active compound. Also in this case the insertion of a propionyl (**2b**) as well as of a benzyl (**2l**) group caused complete loss of activity. This indicates that no substituent or a small alkyl group on the 3 (8) nitrogen, allowing the formation of an ammonium ion, is an essential requirement for the activity of this class. Compound **1a** was also tested in the mouse abdominal constriction test. It demonstrated good protection already at a dose of 5 mg/kg, while at 20 mg/kg no constrictions were observed in the animals (Figure 2). Compound **1a** was then evaluated in comparison with several known analgesics. The antinociceptive effect (hot plate test) induced by icv injection of **1a** (7.5 μg/mouse), nicotine (2 μg), epibatidine (7.5 ng), morphine (7 μg), diphenydramine (10 μg), and clomipramine (30 μg) was greater for **1a** than for the reference drugs, which were all tested at the highest dose that did not impair their rota-rod performance (see Figure 3).

Finally, attempts were made to elucidate its mechanism of action. Administration of naloxone (1 mg/kg ip) did not antagonize antinociception, thus ruling out any involvement of opioid receptors. On the contrary, the prevention of analgesia by the nicotine antagonist mecamylamine (2 mg/kg ip), together with binding studies, suggests the involvement of a nicotinic system.

In Vitro Assays. In binding studies **1a** displayed a K_i of 4.1 ± 0.21 nM (n = 4; [^{3}H]cytisine competition assays). Although potent, it demonstrates a 100-fold lower affinity for the $\alpha_4\beta_2$ nAChR subtype compared to (±)-epibatidine with a K_i of 0.042 ± 0.036 nM (n = 5) (Table 4). nAChR functional activity was also investigated. Three cell lines were examined based on their phenotypic nAChR activities: IMR-32, ganglionic-like; K177, central nervous system; and TE671, neuromuscular junction phenotype. From the Ca^{2+} dynamics induced by **1a** incubation with each cell line, the following EC_{50} values and intrinsic activities (IA, relative to 100 μM (−)-nicotine) were calculated: IMR-32, EC_{50} = 12.2 ± 1.9 μM, IA = 122.6 ± 5.2%; K177, EC_{50} = 24.0 ± 3.1 μM, IA = 164.7 ± 20%; and TE671, EC_{50} > 1000 μM, IA = 9.6 ± 1.6%. (±)-Epibatidine exhibited a high degree of potency and efficacy across all of the cell lines tested (see Table 4). The functional activity of both **1a** and (±)-epibatidine was completely blocked by 100 μM mecamylamine, a specific nAChR antagonist (data not presented).

Modeling. Epibatidine is a quite rigid molecule with the rotation of the chloropyridyl group as the only degree of freedom. This rotation presents a 2-fold minimum; the corresponding conformers have a quite similar energy and are separated by a very low barrier.

Compound **1a** is more flexible due to the presence of the six-membered piperazine ring which can assume a chair or boat conformation and also to the fact that the aryl substituent is linked to the bicyclic system through a nitrogen atom instead of a carbon atom as in the case of epibatidine. So, compound **1a** presents four significant conformations: two chair (**1A,B**) and two boat (**1C,D**) conformations, with **1C** being the global minimum as indicated by calculations. The 3D plots in Figure 4 show that conformer **1C** is also the most similar to epibatidine. Probably, the stability of this last conformer is overestimated as calculations are made in vacuum and a strong hydrogen bond is present between an ammonium hydrogen atom and the 2′ nitrogen atom

Table 2. Effects of Compounds **1** and **2** on Mouse Hot Plate Test[a]

treatment		licking latency (s)			
compd	dose mg/kg sc	before[b] treatment	15 min[b]	30 min[b]	45 min[b]
saline		13.6 ± 0.9 (6)	14.7 ± 1.7 (6)	13.9 ± 1.4 (6)	15.0 ± 1.2 (6)
1a	1	13.5 ± 1.0 (5)	21.5 ± 2.5* (5)	25.6 ± 2.1** (5)	21.5 ± 2.2* (5)
	5	13.7 ± 1.3 (5)	27.8 ± 2.3** (5)	28.5 ± 2.4** (5)	26.5 ± 2.2** (5)
	10	14.2 ± 1.2 (7)	32.3 ± 2.5** (7)	38.3 ± 2.6** (7)	33.0 ± 2.2** (7)
	20	14.3 ± 0.9 (7)	40.3 ± 2.7** (7)	39.8 ± 4.1** (7)	33.8 ± 3.0** (7)
1b	20	13.8 ± 1.2 (6)	17.1 ± 1.9 (6)	15.6 ± 2.7 (6)	18.0 ± 1.8 (6)
	40	14.5 ± 0.8 (11)	17.0 ± 1.8 (11)	16.4 ± 2.0 (11)	18.4 ± 1.0 (11)
1c	10	13.2 ± 1.1 (5)	19.3 ± 1.9* (5)	18.4 ± 1.4 (5)	16.4 ± 1.5 (5)
	25	14.3 ± 1.0 (10)	26.0 ± 2.5** (10)	20.5 ± 1.5* (10)	20.8 ± 1.2* (10)
1d	5	14.7 ± 1.1 (6)	20.3 ± 2.4* (6)	20.5 ± 2.1* (6)	18.7 ± 2.6* (6)
	25	15.1 ± 0.6 (10)	40.8 ± 2.8** (10)	43.8 ± 1.2** (10)	36.5 ± 3.3** (10)
1e	3	14.5 ± 0.9 (5)	21.3 ± 2.0* (5)	17.4 ± 1.5 (5)	16.2 ± 1.5 (5)
	10	15.3 ± 0.8 (9)	31.1 ± 3.4** (9)	26.0 ± 2.5** (9)	24.3 ± 1.9** (9)
1f	5	15.0 ± 1.1 (6)	20.5 ± 1.7* (6)	17.8 ± 1.8 (6)	15.6 ± 2.2 (6)
	10	14.6 ± 0.8 (9)	26.3 ± 2.6** (9)	21.2 ± 2.4** (9)	22.1 ± 3.0** (9)
1g	10	13.6 ± 1.1 (7)	22.2 ± 1.4** (7)	19.7 ± 2.9* (7)	19.6 ± 2.7* (7)
	25	14.9 ± 1.3 (10)	39.5 ± 5.3** (10)	38.5 ± 3.8** (10)	39.1 ± 3.0** (10)
1h	5	15.1 ± 0.9 (8)	19.7 ± 2.2* (8)	15.5 ± 1.8 (8)	15.7 ± 1.3 (8)
	10	13.6 ± 0.6 (10)	24.6 ± 3.1** (10)	22.5 ± 1.5* (10)	22.3 ± 2.2* (10)
1i	5	13.7 ± 0.9 (10)	21.3 ± 2.5* (10)	20.3 ± 2.1* (10)	18.3 ± 2.0 (10)
	25	15.3 ± 0.6 (10)	39.2 ± 2.4** (10)	29.7 ± 3.1** (10)	27.5 ± 3.2** (10)
1j	15	13.5 ± 0.8 (6)	23.6 ± 1.9** (6)	22.1 ± 2.5** (6)	22.0 ± 2.4** (6)
	25	14.6 ± 0.5 (12)	31.9 ± 2.7** (12)	37.0 ± 2.7** (12)	34.2 ± 2.9** (12)
2a	10	12.2 ± 0.4 (5)	17.0 ± 1.7 (5)	15.4 ± 0.5 (5)	17.4 ± 1.2 (5)
	15	15.3 ± 0.8 (5)	23.2 ± 1.6** (5)	22.9 ± 2.7** (5)	20.6 ± 1.8* (5)
	25	15.0 ± 0.9 (5)	35.5 ± 2.5** (5)	34.8 ± 5.8** (5)	26.4 ± 4.5** (5)
2b	10	15.8 ± 0.4 (7)	16.0 ± 0.7 (7)	16.7 ± 1.2 (7)	16.1 ± 0.7 (7)
	50	14.6 ± 0.6 (6)	17 ± 3 ± 0.8 (6)	13.0 ± 0.8 (6)	14.0 ± 0.6 (6)
2l	50	14.8 ± 0.6 (12)	17.2 ± 1.0 (12)	15.3 ± 0.8 (12)	17.6 ± 0.8 (12)

[a] Measured at 52.5 °C as licking latency (s) at various times after treatment. [b] In parentheses is the number of animals; *$P < 0.05$, **$P < 0.01$.

of the aromatic moiety. In solution, in particular in polar solvents, the difference in energy between the boat and chair conformers should be lower and the order of stability could even reverse. Using experimental data diagnostic of the conformation of the piperazine ring, the two vicinal coupling constants between the hydrogen atom at C-1 and the two hydrogen atoms at C-2 have been measured in the ^{1}H NMR spectrum of compound **1a** in D_2O (see Experimental Section). They resulted in 1.5 and 2.8 Hz and are in agreement with the values calculated with the Altona equation[12] for the conformers **1A,B** but not **1C,D**. Thus in aqueous solution the preferred conformers are **1A,B**, though a small contribution of conformer **1C** cannot be excluded.

Compounds **1d**−**j** are quite similar to **1a.** In all the cases, in fact, a nitrogen atom ortho with respect to the carbon atom linked to the bicyclic moiety is present, thus allowing the conformation of type **1C** to become the global minimum.

Compound **2a** shows three allowed conformers: **2A**−**C** (see Figure 4); conformer **2B** presents the same hydrogen bond as **1C** and is the most similar to epibatidine.

Table 3. Comparison between the Minimal Analgesic Dose, Maximal Nontoxic Dose, and Efficacy of **1**, **2**, and the Reference Drugs Morphine and Epibatidine[a]

compd	MAD,[b] mg/kg sc	MNTD, mg/kg sc	% analgesic efficacy compared to morphine	% analgesic efficacy compared to epibatidine
morphine	2	20	100	122
epibatidine	0.001	0.007	82	100
1a	1	30	136	167
1b	inactive	>60		
1c	10	20	61	74
1d	5	30	149	182
1e	3	15	82	99
1f	5	15	60	75
1g	10	30	127	156
1h	5	15	57	70
1i	10	40	124	151
1j	15	30	116	142
2a	15	30	107	130
2b	inactive	>50		
2l[c]	inactive	>50		

[a] Antinociceptive effect was evaluated on the mouse hot plate test. The maximal analgesic effect of morphine or epibatidine is indicated as 100%. [b] Minimal dose able to induce a statistically significant increase of the pain threshold. [c] Administered po.

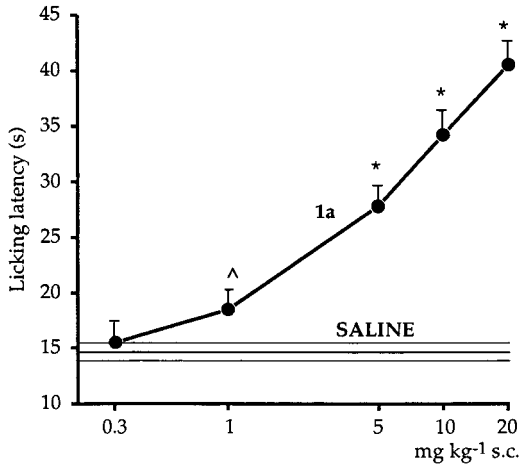


Figure 1. Dose−response curve of **1a** in the mouse hot plate test. **1a** was administered 15 min before the test. Vertical lines show SEM; $^P < 0.05$, $*P < 0.01$ in comparison with saline controls. Each point represents the mean of at least 8 mice.

The experimental values of the coupling constants between H-1 and H_2-2 are in agreement with the chair conformation of the piperazine ring (**2A**) indicating that also in this case calculations in vacuum overestimate the hydrogen bond.

Conclusions

In conclusion, 3-(6-chloro-3-pyridazinyl)-3,8-diazabicyclo[3.2.1]octane (**1a**) and several of its analogues proved to be interesting analgesics, fully comparable to morphine in their potency, though acting through a nonopiate mechanism. In comparing the functional profiles of **1a** and (±)-epibatidine, the following characteristics have surfaced: (*a*) **1a** binds with high affinity to the central nAChR subtype, $\alpha_4\beta_2$, albeit with 100-fold lower potency than (±)-epibatidine; *(b)* **1a** is a full agonist at both the ganglionic (IMR-32 cells) and central (K177 cells) nAChR subtypes exhibiting a narrow separation between potencies at these receptors (2-fold difference in EC_{50} values); and *(c)* unlike (±)-epibatidine, **1a** did not stimulate the nAChR subtypes expressed in the

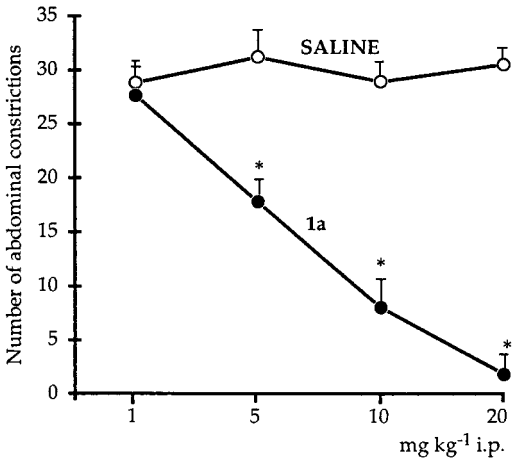


Figure 2. Dose−response curve of **1a** in the mouse abdominal constriction test. **1a** was administered 15 min before the test. Vertical lines show SEM. $*P < 0.01$ in comparison with saline controls. Each point represents the mean of at least 8 mice.

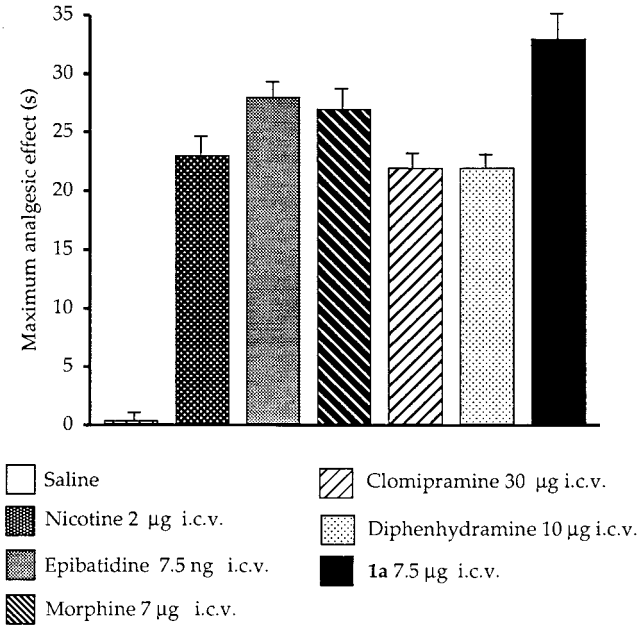


Figure 3. Maximum antinociceptive effect of **1a** in comparison with nicotine, epibatidine, morphine, diphenhydramine, and clomipramine evaluated in the mouse hot plate test. The nociceptive responses were recorded 5 min after nicotine icv injection, 15 min after icv administration of epibatidine, diphenhydramine and clomipramine, and 30 min after icv morphine injection. Each column represents the mean of at least 10 mice. Vertical lines show SEM.

TE671 cell line. Thus, the antinociceptive properties of **1a** appear to be nAChR-mediated.[13] Moreover, **1a** has the potential of producing antinociceptive effects in vivo without the potential life-threatening side effects presented by (±)-epibatidine (i.e., cardiovascular pressor effects and seizures)[14] due to the lack of agonist activity at the neuromuscular junction. Finally, the results of the modeling indicate that **1a** presents one conformation (**1C**) similar to that of epibatidine. Thus, in the hypothesis that **1a** competes for the same nicotinic receptor as epibatidine, **1C** could be the bioactive conformer, even if, according to NMR data, it is not present in polar solvents.

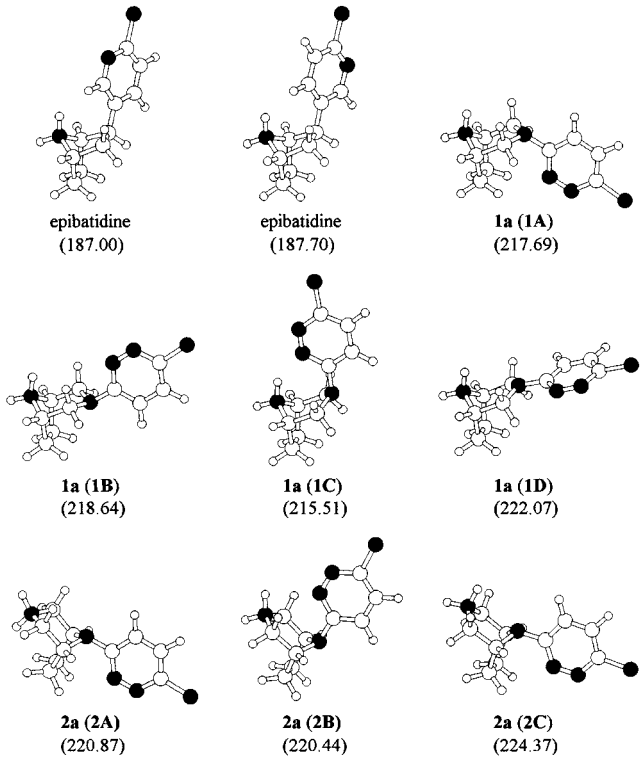


Figure 4. Three-dimensional plots of the significant conformers of epibatidine and of compounds **1a** and **2a**. In parentheses are the heats of formation (ΔH_f, kcal/mol).

Table 4. Cholinergic Interactions of **1a** and (±)-Epibatidine[a]

assay	**1a**	(±)-epibatidine
[^{3}H]cytisine		
K_i, μM	4.1 ± 0.21	0.042 ± 0.036
FLIPR,[b] IMR-32		
EC_{50}, μM	12.2 ± 1.9	0.034 ± 0.003
IA,[c] %	122.6 ± 5.2	145.6 ± 23
FLIPR, K177		
EC_{50}, μM	24.0 ± 3.1	0.35 ± 0.02
IA, %	164.7 ± 20	148.1 ± 17
FLIPR, TE671		
EC_{50}, μM	>1000	1.26 ± 0.11
IA, %	9.6 ± 1.6	79.5 ± 13

[a] Rat cerebral cortical membranes. See also the Experimental Section. [b] Fluorescence imaging plate reader. [c] Intrinsic activity, relative to 100 μM (−)-nicotine.

Experimental Section

Chemistry. Melting points were determined on a Büchi 510 capillary melting point apparatus and are uncorrected. Analyses indicated by the symbols were within ±0.4 of the theoretical values. ^{1}H NMR spectra were recorded on a Bruker AC200 or Bruker AM500 spectrometer; chemical shifts are reported as δ (ppm) relative to tetramethylsilane. TLC on silica gel plates was used to check product purity. Silica gel 60 (Merck; 70−230 mesh) was used for column chromatography.

General Procedure for the Synthesis of the 3-Heteroaryl-Substituted Diazabicyclooctanes 1a−j. An equimolar mixture of 3-benzyl-3,8-diazabicyclo[3.2.1]octane **3**[6] (1 g; 5 mmol) and di-*tert*-butyl dicarbonate (1.1 g) in anhydrous dichloromethane (10 mL) was stirred overnight under nitrogen at room temperature. The solvent was evaporated and the residue purified by flash chromatography (eluent cyclohexane/ethyl acetate, 98/2) to give 3-benzyl-8-(*tert*-butoxycarbonyl)-3,8-diazabicyclo[3.2.1]octane (**4**) (1.39 g, 92%; mp = 60−61 °C). **4** was dissolved in ethanol (10 mL), to the solution 10% Pd−C (0.14 g) was added, and the mixture was hydrogenated at room temperature and external pressure. After the uptake of hydrogen ceased, the catalyst was filtered off, the solvent evaporated, and the residue (8-(*tert*-butoxycarbonyl)-3,8-diazabicyclo[3.2.1]octane, **5**; 0.92 g, yield 95%; mp = 58−61 °C) dissolved in toluene (20 mL). To the solution an equimolar amount of the appropriate heteroaryl derivative was added together with triethylamine (0.61 mL), and it was refluxed for 8 h. After cooling, the precipitate was filtered off, the solvent evaporated, and the residue purified by flash chromatography (eluent dichloromethane/ethyl acetate, 9/1). The so obtained 3-heteroaryl-8-(*tert*-butoxycarbonyl)-3,8-diazabicyclo[3.2.1]-octanes **6** (yield 50−75%) were finally deprotected by treatment with a solution of hydrochloric acid in diethyl ether to give the desired **1a,d−j**, as their hydrochlorides. Compounds **1b,c** were easily obtained from **1a**, according to standard procedures (see Table 1 and Scheme 1).

For **1a** (as the hydrochloride): ^{1}H NMR (D_2O, 500 MHz) δ 1.90 (m, 2H), 2.01 (m, 2H), 3.39 (dd, 2H, J = 14.5, 1.5 Hz), 4.01 (dd, 2H, J = 14.5, 2.8 Hz), 4.17 (m, 2H), 7.41 (d, 1H, J = 9.5 Hz); 7.55 (d, 1H, J = 9.5 Hz)

8-Heteroaryl-Substituted Diazabicyclooctanes 2a,b. Method a. An equimolar amount of 3-propionyl-3,8-diazabicyclo[3.2.1]octane (**7**)[7] (0.5 g, 3 mmol), 3,6-dichloropyridazine (0.45 g), and triethylamine (0.41 mL) in toluene (20 mL) was refluxed for 6 h. After cooling, the precipitate was filtered off, the solvent evaporated, and the residue purified by flash chromatography (cyclohexane/ethyl acetate, 95/5) to give the 3-propionyl-8-(6-chloro-3-pyridazinyl)-3,8-diazabicyclo-[3.2.1]octane (**2b**). **2b** was then hydrolyzed by 4 N NaOH (5 mL) at 60 °C to 8-(6-chloro-3-pyridazinyl)-3,8-diazabicyclo-[3.2.1]octane (**2a**). The corresponding hydrochloride was prepared by hydrochloric acid in diethyl ether (see Table 1 for data)

For **2a** (as the hydrochloride): ^{1}H NMR (D_2O, 500 MHz) δ 1.90 (m, 2H), 2.21 (m, 2H), 3.26 (dd, 2H, J = 13.0, 1.7 Hz), 3.28 (dd, 2H, J = 13.0, 2.3 Hz), 4.75 (m, 2H), 7.50 (d, 1H, J = 9.5 Hz);,7.62 (d, 1H, J = 9.5 Hz).

Method b. An equimolar amount of 3-propionyl-3,8-diazabicyclo[3.2.1]octane (**7**)[7] (0.7 g, 4.2 mmol), benzyl chloride (0.53 g), and K_2CO_3 (0.57 g) in acetone (10 mL) was refluxed overnight. After cooling, the solid was filtered off, the solvent evaporated, and the residue purified by flash chromatography (cyclohexane/ethyl acetate, 95/5) to give 3-propionyl-8-benzyl-3,8-diazabicyclo[3.2.1]octane (**8**) (0.97 g, yield 90%). **8** was hydrolyzed by refluxing with 4 N HCl (3 mL) for 3 h; the mixture was cooled, brought to pH 10, and extracted with CH_2Cl_2 (3 × 20 mL). After evaporation of the solvent the so obtained 8-benzyl-3,8-diazabicyclo[3.2.1]octane (**9**) (0.73 g, 95%) was treated with an equimolar amount of di-*tert*-butyl dicarbonate (0.79 g) in anhydrous dichloromethane (10 mL) and stirred overnight at room temperature under nitrogen. The solvent was evaporated and the residue purified by flash chromatography (eluent cyclohexane/ethyl acetate, 98/2) to give compound **10** (1.06 g, 97%). It was dissolved in ethanol (10 mL). To this was added 10% Pd−C (0.1 g), and the mixture was hydrogenated at room temperature and external pressure. After the uptake of hydrogen ceased, the catalyst was filtered off and the solvent evaporated to give 3-(*tert*-butoxycarbonyl)-3,8-diazabicyclo[3.2.1]octane (**11**) in quantitative yield (0.74 g). Condensation of the latter with 3,6-dichloropyridazine was performed as previously described for **2b**, though a longer time was required (24 h), to give 3-(*tert*-butoxycarbonyl)-8-(6-chloro-3-pyridazinyl)diazabicyclo[3.2.1]octane (**12**) (85%), which was purified by flash chromatography (eluent CH_2Cl_2/ethyl acetate, 9/1) and finally converted into 8-(6-chloro-3-pyridazinyl)diazabicyclo[3.2.1]octane (**2a**) (as its hydrochloride) in quantitative yield, by a solution of hydrochloric acid in diethyl ether.

3-Benzyl-8-(6-chloro-3-pyridazinyl)diazabicyclo[3.2.1]-octane (**2l**) was easily obtained from **3** by refluxing with equimolar amounts of 3,6-dichloropyridazine and triethylamine in toluene for 6 h (yield 65%).

Pharmacology. Morphine (hydrochloride) was obtained from USL 10D (Florence, Italy). Clomipramine (hydrochloride) was purchased from Ciba Geigy (Basel, Switzerland) and

diphenhydramine (hydrochloride) from De Angeli (Milan, Italy). (±)-Epibatidine was obtained from Research Biochemicals International (Natick, MA). (−)-Nicotine (hydrogen tartrate salt) and mecamylamine (hydrochloride) were purchased from Sigma Chemical Co. (St. Louis, MO). [^{3}H]-(−)-Cytisine was obtained from NEN Life Sciences (Boston, MA).

In Vivo Experiments. Analgesic activity was evaluated in male Swiss-Webster mice (22−28 g; Morini, San Polo d'Enza, Italy). Mice were kept at 23 ± 1 °C, with a 12-h light/dark cycle, light at 7 a.m., with food and water ad libitum. All experiments were carried out according to the guidelines of the European Community Council on animal care.

Hot plate method described by O'Callaghan and Holtzman[15] was used to assess the potential analgesic activity of the test compounds. The plate temperature was fixed at 52.5 ± 0.1 °C. Mice with a licking latency below 12 and over 18 s in the test before drug administration (30%) were rejected. An arbitrary cutoff time of 45 s was adopted. The number of mice treated in each test varied from 5 to 12. To evaluate the analgesic efficacy of the new products, their level of analgesia reached after the injection of the maximal dose unable to modify mouse rota-rod performance was compared to that of morphine (8 mg/kg sc) or epibatidine (5 μg/kg sc), taken as reference compounds. The integrity of motor coordination was assessed on the basis of the endurance time of the animals on the rotating rod, according to Vaugh.[16] Analgesic efficacy of each compound (**X**) is expressed as percentage of that of epibatidine (5 μg/kg sc) or morphine (8 mg/kg sc). The calculation was performed using the following formula:

$$\% \text{ of analgesic efficacy of } \mathbf{X} = (T_1 - T_2)/(T_3 - T_4) \times 0.100$$

where T_1 is maximum reaction time of **X**, T_2 is pretest reaction time of **X**, T_3 is maximum reaction time of morphine or epibatidine, and T_4 is pretest reaction time of morphine or epibatidine.

The maximal nontoxic dose is considered the highest dose of **X** which does not cause any visible change in animal behavior such as tremors, convulsions, hypomotility, etc., so that the researchers were unable to distinguish between treated and untreated mice. The evaluation of the behavioral parameters was performed according to Irwing[17] on a group of at least 10 animals. The abdominal constriction test was performed according to Koster et al.[18] Standard errors on the values expressed as percentage were not evaluated. Original data, however, have been statistically processed by employing Dunnett's two-tailed test in order to verify the significance of the differences between the means shown by treated mice at the maximum reaction time and the pretest reaction time. Differences were considered statistically significant when $P < 0.05$. Percent values were calculated only for those differences that were statistically significant. In other case, drugs were considered inactive. Intracerebroventricular (icv) administration was performed under ether anesthesia using isotonic saline as a solvent, according to the method described by Haley and McCormick.[19]

In Vitro Experiments. 1. Membrane Preparations: Rat cerebral cortical membranes were purchased from ABS Inc. (Wilmington, DE). Prior to use, the frozen membrane pellets were slowly thawed, washed, and resuspended in 30 volumes of assay buffer (composition, mM: Tris HCl, 50; NaCl, 120; KCl, 5; $MgCl_2$, 1°, and $CaCl_2$, 2.5; pH 7.4 at 4 °C). The homogenate was centrifuged at 45000*g* for 20 min at 4 °C and the pellet resuspended in ice-cold buffer.

2. [^{3}H]-(−)-Cytisine Binding: Binding conditions were as previously described.[20] Samples containing 150−200 μg of protein, 0.7 nM [^{3}H]-(−)-cytisine (30 Ci/mmol), and the various concentrations of the nAChR modulators were incubated in a final volume of 500 μL for 75 min at 4 °C in triplicate. Nonspecific binding was determined in the presence of 10 μM (−)-nicotine.

3. Tissue Culture: Cells of the IMR-32 human neuroblastoma clonal line (ATCC, Rockville, MD) were maintained in a log phase of growth according to established procedures.[21] HEK-293 cells (ATCC, Rockville, MD) stably expressing the human $\alpha_4\beta_2$ nAChR subunit combination, K177 cells, were maintained as previously described.[22] TE671 cells (ATCC, Rockville, MD) were grown according to the protocol of Lukas.[23] Cells were plated out at a density of 1×10^{-6} cells/well on black-walled, clear-bottomed 96-well plates and used approximately 72 h after plating. All plates were coated with poly(ethylenimine) to aid in the adherence of the cells to the plate.

4. nAChR Functional Activity: Changes in the intracellular free Ca^{2+} concentrations were measured using the calcium-chelating dye Fluo-3 (Molecular Probes, Eugene, OR) in conjunction with a FLIPR (Molecular Devices, Sunnyvale, CA). The cell permeant acetoxymethyl (AM) ester form of Fluo-3 was prepared to a concentration of 1 mM in anhydrous DMSO and 10% pluronic acid. The dye was then diluted to a final concentration of 4 mM in growth media and placed on the cells for 1 h at 37 °C. Black-walled 96-well plates were utilized to reduce light scattering. The unincorporated dye was removed from the cells by excessive washing with Hepes-salts buffer (composition, mM: Hepes, 20; NaCl, 120; KCl, 5; $MgCl_2$, 1; glucose, 5; and $CaCl_2$, 5; pH 7.4 at 25 °C). After addition of various concentrations of agonists, the Ca^{2+} dynamics were observed in the FLIPR apparatus equipped with an argon laser (wavelength, 480 nm), an automated 96-channel pipettor, and a CCD camera. The intensity of the fluorescence was captured by the CCD camera every second for the first minute following the agonist addition with additional readings every 5 s for a total time period of 5 min. These images were digitally transferred to an interfaced PC and changes in fluorescence intensity processed for each well. The exposure setting of the camera was 0.4 s with *f* stop setting of 2 μm. The percent maximal intensity relative to that induced by 100 μM nicotine was plotted against the concentration of agonist, and EC_{50} values were calculated. Independent measurements of 100 μM nicotine (100%) and unloaded cells (0%) were performed on each plate of cells with an average range of 20 000 fluorescence units. These controls allowed for normalization across several plates of cells.

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Supporting Information Available: Tables of ^{1}H NMR data, effects of **1** and **2** in the rota-rod test, and results of modeling (3 pages). See any current masthead page for ordering information.

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(24) The structure of compounds **1e,f** has been assigned on the basis of NOE experiments, performed at 500 MHz in D_2O on their hydrochlorides, which showed for **1e** the enhancement of H-2_{eq}/H-4_{eq} (5%) on irradiation of the aromatic proton at δ 6.81 (H-5′) and, vice versa, the enhancement of H-5′ (16%) on irradiation of the signal at δ 4.32 (H-2_{eq}/H-4_{eq}). On the contrary, no significant enhancement was observed on irradiation at the aromatic hydrogen atoms at δ 6.75 and 8.10 of **1f**.

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